

**EXPRESS MAILING CERTIFICATE**"EXPRESS MAIL" Mailing Label No.: EL085326385USDate of Deposit : February 13, 2002

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231

Typed or printed name of person signing this certificate:

Signed: Bonnie Harbin-Mitchell  
Bonnie HARBIN-Mitchell

**A PROTEIN THAT ENHANCES EXPRESSION OF POTASSIUM CHANNELS ON  
CELL SURFACES AND NUCLEIC ACIDS THAT ENCODE THE SAME**

**Background of the Invention**

The electrical properties of excitable cells are determined in large part by the voltage-gated  $K^+$  channels, i.e., "Kv channels", present on the plasma membrane of such cells. Kv channels are also important in many nonexcitable cells where they contribute to diverse processes such as volume regulation, hormone secretion, and activation by mitogens. At least 50 different Kv channel genes have been identified, and most have been assigned to one of the following four major subfamilies: Kv1, Kv2, Kv3, and Kv4. Each Kv channel gene encodes a single pore-forming subunit, referred to as the  $\alpha$ -subunit. Functional Kv channels are formed by the tetrameric association of individual  $\alpha$ -subunits. With multiple  $Kv\alpha$  proteins that assemble as multi-subunit heteromeric complexes, there may be hundreds of functionally distinct Kv channels.

Kv channels, either functioning or malfunctioning, are implicated in many disease states including cardiac arrhythmias, hypertension, angina, asthma, diabetes, renal insufficiency, urinary incontinence, irritable colon, epilepsy, cerebrovascular ischemia and autoimmune diseases. Accordingly, efforts are underway to identify and characterize pharmacological agents that alter the kinetics, gating or formation of Kv channels. The efficacy of such agents is determined by treating cells with such agents and measuring changes in current across the plasma membrane of the cells. Unfortunately, it is difficult to measure small changes in the current in most cells. It is also difficult to determine whether a pharmacological agent alters current flow through a specific Kv channel. Accordingly, it is desirable to have methods and tools which can be used to regulate the numbers and types of Kv channels on the plasma

membrane of cells. It is also desirable to have new research tools that can be used for examining the assembly and synthesis of Kv channels.

### **Summary of the Invention**

5 The present invention provides novel polynucleotides that encode a novel protein, designated herein as K<sup>+</sup> Channel Associated Protein or "KChAP". It has been determined that expressing polynucleotides that encode KChAP in host cells, along with polynucleotides that encode the Kv $\alpha$  channel subunit Kv 2.1, the Kv $\alpha$  channel subunit Kv 2.2, the Kv $\alpha$  channel subunit Kv 1.3, or the Kv $\alpha$  channel subunit Kv 4.3, increases the number of Kv2.1, Kv 2.2, Kv1.3 or Kv4.3 channels, respectively, in the plasma membrane of such cells. Accordingly, KChAP polynucleotides are useful for making cells that have increased numbers of Kv channels on the cellular plasma membrane. Such cells are useful model systems for studying the effect of pharmacological agents on Kv channels, particularly on Kv2.1, Kv 2.2, Kv 1.3, and Kv 4.3 channels.

10 The present invention also relates to the novel protein KChAP. During formation of Kv channels, KChAP binds to the Kv $\alpha$  channel subunits Kv2.1, Kv2.2, Kv1.3, and Kv4.3 within the cytoplasm of the cell. KChAP also binds to the Kv $\alpha$  channel subunits Kv1.2, Kv 1.4, Kv1.5 and Kv 3.1 and to Kv $\beta$  subunits. Accordingly, KChAP and the antibodies that are immunospecific for KChAP are useful research tools for monitoring the interaction between diverse Kv $\alpha$  channel subunits and KChAP and for monitoring the interaction between Kv $\alpha$  subunits and Kv $\beta$  subunits.

### **Brief Description of the Figures**

25 Figure 1 is a schematic representation of a full-length cDNA that encodes KChAP and partial fragments thereof. The open reading frame is flanked by 219 base pairs of untranslated sequence on the 5' end, and 980 base pairs of untranslated sequence on the 3' end as indicated by the thin lines. KChAP-Y depicts the partial clone that was originally isolated in the yeast two-hybrid screen. KChAP-Y extends from amino acid W310 through the poly A tail at the 3' end. The domain on KChAP that binds to Kv $\alpha$  subunits and to Kv $\beta$  subunits, hereinafter referred to as the "Kv $\alpha$ /Kv $\beta$ " binding domain", has been localized to the region between amino acids W310 and L407 ;

Figure 2 shows a cDNA sequence, SEQ ID NO: 1, that encodes rat KChAP, and the predicted amino acid sequence, SEQ ID NO: 2, of the KChAP protein encoded by the rat cDNA;

Figure 3 shows a cDNA sequence, SEQ ID NO: 3, that encodes human KChAP, and the predicted amino acid sequence, SEQ ID NO: 4, of the KChAP protein encoded by the human cDNA;

Figure 4 provides a comparison of the amino acid sequences of rat KChAP and human KChAP. The double dots between the sequences identify highly conserved amino acids, i.e., amino acids that are similar in size, hydrophobicity, and charge. The single dot between the aligned amino acid sequences identify amino acids that are less highly conserved.

### Detailed Description of the Invention

#### The KChAP Protein

The present invention provides a unique protein KChAP. The mature form of KChAP has a calculated molecular weight of about 62.4 kDa. In one embodiment the human KChAP protein has the amino acid sequence shown in FIG. 3 (SEQ ID NO: 4). In one embodiment the rat KChAP protein has the amino acid sequence shown in FIG. 2 (SEQ ID NO: 2).

The present invention also relates to allelic variants or derivatives of the amino acid sequences shown in Figs. 2 and 3. In addition to naturally occurring allelic forms of the protein, the KChAP protein as described herein embraces non-naturally occurring derivatives of the KChAP protein where one or more of the amino acids have been replaced by conservative amino acid residues, typically by using direct synthesis or recombinant techniques. The present invention also relates to allelic variants or derivatives of the KChAP that have an amino acid sequence identity of at least 85%, more preferably at least 90%, and most preferably of at least 95% with the amino acid sequences shown in Fig. 2 or Fig. 3, provided however, that the derivative is capable of binding to the N-termini of the Kv $\alpha$  subunits Kv 2.1, Kv2.2, Kv 1.3, Kv4.3 and to the C-terminus of Kv $\beta$  1.2.

In another aspect, the present invention relates to an isolated peptide which comprises the domain of KChAP that binds to Kv $\alpha$  subunits, particularly the Kv $\alpha$  subunits Kv 2.1, Kv2.2, Kv4.3 and to Kv $\beta$  subunits, particularly Kv $\beta$  1.2. Such domain is hereinafter referred to as the "Kv $\alpha$ /Kv $\beta$  binding domain". As used herein, peptide means a fragment of the KChAP protein

and accordingly is smaller and comprises fewer amino acids than the KChAP protein. In one embodiment, this peptide comprises the amino acid sequence, SEQ ID NO: 5, extending from T309 through L407 as shown in Figure 2 and the amino acid sequence, SEQ ID NO: 7, extending from T309 through L407 as shown in Fig. 3. The present invention also relates to  
 5 allelic variants or derivatives of the amino acid sequence set forth in SEQ ID NO's: 5 and 7.

In another aspect, the present invention relates to an isolated peptide which comprises the C terminal domain of the KChAP protein, i.e., the last 160 to 170 amino acids of the KChAP protein. In one embodiment, the peptide comprises the amino acid sequence set forth in SEQ ID NO: 8. In another embodiment, the peptide comprises the amino acid sequence set forth in SEQ.  
 10 ID NO: 9. In another embodiment, the peptide is a variant of the C terminal domain of the rat KChAP protein and the human KChAP protein and comprises:

(a) a K $\alpha$ /K $\beta$  binding domain having the following sequence:

WTCPVCDKKA PYESLIIDGL FMEILXaSCSD CDEIQFMEDG

SWCPMKPKKE ASEVCPPPGY GLDGLQYSPV QXaGXaXaSENKK

XaVEVIDLTIE SSSDEEDL, SEQ ID NO: 10,

wherein the Xa at position 25 is serine or asparagine,

the Xa at position 72 is glycine or glutamic acid,

the Xa at position 74 is aspartic acid or asparagine,

the Xa at position 75 is proline or glutamine

the Xa at position 80 is lysine or arginine; and

(b) a C terminal domain having the following sequence:

PPTKKHCXaVT SAAIPALPGS KGXaLTSGHQPS SSVLRSPAMG

TL GXaDFLSSL PLHEYPPAFP LG ADIQGLDL FSFLQTESQH

YXaPSVITSLD EQDXaLGHFFQ XaRGTPXaHFLG PLAPTLGSSH

XaS ATPAPXaPG RVSSIVAPGXa XaLREGHGGPL PSGPSLTGCR

SDIXaSLD SEQ ID NO: 11;

wherein the Xa at position 8 is serine or proline,

the Xa at position 23 is valine or alanine,  
the Xa at position 44 is glycine or serine,  
the Xa at position 82 is, glycine or serine,  
the Xa at position 94 is alanine or threonine,  
the Xa at position 101 is tyrosine or phenylalanine,  
the Xa at position 106 is serine or proline,  
the Xa at position 121 is cystine or arginine,  
the Xa at position 128 is proline or alanine,  
the Xa at position 140 or glycine or serine,  
the Xa at position 141 is alanine or serine,  
the Xa at position 164 is isoleucine or valine.

Such peptides are useful for producing antibodies that are immunospecific for KChAP.

The present invention also relates to fusion proteins wherein additional amino acids are fused to the KChAP protein or to the peptide fragments of KChAP. The additional amino acids are added at either the 3' end or 5' end of the protein or peptide, for example, to aid in purification of the protein or peptide. The KChAP proteins and peptides are provided in an isolated form.

KChAP is not a channel protein. KChAP binds with the N-termini of Kv $\alpha$ 1 and Kv $\alpha$ 2 subunits. Specifically, KChAP binds with the  $\alpha$  subunits Kv 2.1, Kv 2.2, Kv 1.3, Kv 4.3, Kv1.2, Kv1.4, Kv1.5. KChAP also binds to Kv $\beta$  subunits, particularly Kv $\beta$ 1 and its isoforms. Kv $\beta$  subunits are cytoplasmic proteins that form stable complexes with Kv $\alpha$ 1 subunits. Kv $\beta$  subunits are strong modulators of Kv channels. The Kv $\beta$  subunit, Kv $\beta$ 1.2 suppresses current in the Kv1.5 potassium channel; this effect is abolished by KChAP which binds the Kv $\beta$ 1.2.

#### Preparing KChAP

KChAP may be synthetically produced by conventional peptide synthesizers. Preferably, KChAP is produced using cell-free translation systems and RNA molecules derived from DNA

constructs that encode the KChAP protein. Alternatively, KChAP is made by transfecting host cells with expression vectors that comprise a DNA sequence that encodes the KChAP protein and then inducing expression of the protein in the host cells. For recombinant production, recombinant constructs comprising one or more of the sequences which encode KChAP are introduced into host cells by conventional methods such as calcium phosphate transfection, DEAE-dextran mediated transfection, transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape lading, ballistic introduction or infection.

KChAP is expressed in suitable host cells, such as for example, mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters using conventional techniques. Following transformation of the suitable host strain and growth of the host strain to an appropriate cell density, the cells are harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification of the KChAP protein.

Conventional procedures for isolating recombinant proteins from transformed host cells, such as isolation by initial extraction from cell pellets or from cell culture medium, followed by salting-out, and one or more chromatography steps, including aqueous ion exchange chromatography, size exclusion chromatography steps, high performance liquid chromatography (HPLC), and affinity chromatography may be used to isolate recombinant KChAP.

#### Preparation of Antibodies

Recombinant KChAP or portions thereof, i.e., KChAP peptides, are used as immunogens to produce antibodies immunospecific for wild-type KChAP. Preferably, the KChAP peptides have little sequence homology with the human Gu binding protein, whose amino acid sequence is about 50% homologous with the amino acid sequences shown in Figs 2 and 3. The term “immunospecific” means the antibodies have substantially greater affinity for KChAP than for other proteins. Such antibodies are generated using conventional techniques by administering KChAP or the portion thereof to an animal, preferably a nonhuman, more preferably a rabbit. Conventional protocols are also used to collect blood from the immunized animals and to isolate the serum and or the IgG fraction from the blood. For preparation of monoclonal antibodies, conventional hybridoma techniques are used.

Polyclonal serum to KChAP was made using a bacterial fusion protein comprising the C-terminal 167 amino acids of KChAP. i.e., from amino acid L407, fused to the maltose binding protein. The fusion protein was prepared by subcloning the C-terminal 167 amino acids of KChAP into pMAL-C2 vector from New England Biolabs. The resulting vector was used to transform E. coli. The fusion protein was isolated from transformed E. coli cells on an amylose resin and sent to Research Genetics, Inc. for generation of polyclonal sera using conventional techniques

Antibodies to KChAP are useful analytical tools for monitoring the formation of Kv channels and for studying the intracellular association of KChAP with Kv $\alpha$  subunits and with Kv $\beta$  subunits. Such antibodies are also useful reagents for identifying the intracellular location of the interaction between KChAP and Kv $\alpha$  and Kv $\beta$  subunits. Such antibodies are also useful to isolate or identify cells expressing the KChAP protein and to purify KChAP from partially purified preparations by affinity chromatography.

#### The KChAP Polynucleotide

The present invention also provides polynucleotides that encode the KChAP protein and the KChAP peptides of the present invention, hereinafter referred to collectively as the "KChAP polynucleotides". The KChAP polynucleotide is single stranded or double stranded. The polynucleotide is a DNA or RNA molecule, preferably a DNA molecule, and comprises a sequence which codes for the KChAP protein, preferably the human KChAP protein, or fragments thereof. Optionally, the polynucleotide also comprises a leader sequence and encodes a KChAP protein which is processed and secreted from mammalian cells as the mature polypeptide. Polynucleotides encoding KChAP protein may also be fused in frame to a marker sequence which allows for purification of the KChAP protein such as the maltose binding protein, which binds to amylose resin. Polynucleotides encoding KChAP protein or KChAP peptide fragments may also be fused in frame to a marker sequence, such as c-myc, which encodes an epitope that allows for monitoring the intracellular location of KChAP using commercially available antibodies.

In one embodiment, the KChAP polynucleotide encodes for a KChAP protein comprising the amino acid sequence shown in FIG. 2, SEQ ID NO: 2. One example of a polynucleotide that encodes the protein of SEQ ID NO: 2, is depicted in FIG. 2, and set forth in SEQ ID NO: 1. In another embodiment, the polynucleotide encodes for a KChAP protein comprising the amino

acid sequence shown in FIG. 3, SEQ ID NO: 4. One example of a polynucleotide that encodes the protein of SEQ ID NO: 4 is depicted in FIG. 3 and set forth in SEQ ID NO: 3. The present invention also relates to polynucleotides that encode an allelic variant of the proteins having the amino acid sequences shown in Figs. 2 and 3.

5 ~~Sub 927~~ In another embodiment, the polynucleotide encodes for variants of KChAP protein, wherein the variants have the following sequence:

MKIKELYRRR FPRKTLGPSD LSLSLPPGT SPVGSPXaPLA XaIPPTLLXaPG  
 TLLGPKREVD MHPPLPQPVH PDVTMKPLPF YEYVGELIRP TTLASTSSQR;  
 FEEAHFTFAL TPQXXaQQILT SREVLPGAKC DYTIVQQLRF CLCETSCPQE;  
 10 DYFPPNLFVK VNGKLCPLPG YLPPTKNGAE PKRPSRPINI TPLARLSATV;  
 PNTIVVNWSS EFGRNYSLSV YLVRQLTAGT LLQKLRAKGI RNPDHSRALI;  
 KEKLTADPDS EVATTSLRVS LMCPLGKMRL TVPCRALTCA HLQSFDAALY;  
 LQMNEKKPTW TCPVCDKKAP YESLIIDGLF MEILXaSCSDC DEIQFMEDGS;  
 WCPMKPKKEA SEVCPPPGYGLDGLQYSPVQ XaGXaPSENKKXa VEVIDLTIES;  
 SSDEEDLPP TKKHXCXaVTSA AIPALPGSKG XaLTSGHQPS VLRSPAMGTLG;  
 XaDFLSSLPLH EYPPAFPLGA DIQGLDLFSF LQTESQHYXaP SVITSLDEQD;  
 XaLGHFFQXaRG TPXaHFLGPLA PTLGSSHXaSA TPAPXaPGRVS SIVAPGXaXaLR;  
 EGHGGPLPSG PSLTGCRSDI XaSLD, SEQ ID NO: 6;

wherein the amino acid Xa at position 37 is glycine or serine;

20 the amino acid Xa at position 41 is proline or serine;

the amino acid Xa at position 48 is alanine or threonine;

the amino acid Xa at position 115 is valine or leucine;

the amino acid Xa at position 335 is serine or asparagine;

the amino acid Xa at position 381 is glycine or glutamic acid;

25 the amino acid Xa at position 383 is aspartic acid or asparagine;

the amino acid Xa at position 384 isoproline or glutamine;

the amino acid Xa at position 390 is lysine or arginine;

the amino acid Xa at position 416 is serine or proline;



the amino acid Xa at position 431 is valine or alanine;  
 the amino acid Xa at position 451 is glycine or serine;  
 the amino acid Xa at position 489 is glycine or serine;  
 the amino acid Xa at position 501 is alanine or threonine;  
 5 the amino acid Xa at position 508 is tyrosine or phenylalanine;  
 the amino acid Xa at position 513 is serine or proline;  
 the amino acid Xa at position 528 is cysteine or arginine  
 the amino acid Xa at position 535 is proline or alanine;  
 the amino acid Xa at position 547 is glycine or serine;  
 10 the amino acid Xa at position 548 is alanine or serine;  
 the amino acid Xa at position 570 is isoleucine or valine.

The present invention further relates to polynucleotides which are complementary to sequences that have at least 85% identity, preferably 90% identity, more preferably 95% identity with the nucleotide sequences which encode the amino acid sequences shown in Figs. 2 and 3 or SEQ ID NO: 6.

Preferably, the polynucleotides comprise a sequence which hybridizes under stringent conditions to sequences which encode the amino acid sequence shown in Fig 2 and Fig. 3 or sequences which are complementary thereto. As herein used, the term "stringent conditions" means hybridization will occur if there is at least 95% and, preferably, at least 97% identity between the sequences. Preferably, the polynucleotide is provided in an isolated form.

The polynucleotides that encode the KChAP protein are useful for preparing cells that have increased numbers of Kv channels on their cell surface. The polynucleotides of the present invention are useful for preparing cells that have Kv channels formed from exogenous Kv $\alpha$  subunits. As used herein, "an exogenous Kv $\alpha$  subunit " means that the gene encoding the Kv $\alpha$  subunit is not normally expressed in the cell. Kv $\alpha$  subunits that are normally expressed in a cell are referred to as endogenous subunits. To prepare the cells, polynucleotides encoding KChAP and a Kv $\alpha$  subunit, an exogenous Kv $\alpha$  subunit, preferably are co-transfected or co-injected into host cells. Preferably, the cRNA molecules that encode KChAP and the Kv $\alpha$  subunit are coinjected with one pipette. Preferably, the Kv $\alpha$  subunit is a Kv2.1, Kv2.2, Kv1.3 or Kv4.3 subunit, The resulting cells, which have on the surface thereof increased numbers of Kv channels formed by the exogenous Kv $\alpha$  subunits, are useful for testing the efficacy of

compounds designed to alter current flow through the newly-expressed Kv channels such as, for example by measuring whole-cell currents using the conventional two microelectrode voltage-clamp technique.

The KChAP polynucleotides are also useful for producing KChAP constructs which are useful for producing KChAP protein or fragments thereof by recombinant techniques. Such constructs include, among others, vectors, such as a plasmid, phagemid, or viral vector, into which a sequence that encodes the KChAP protein has been inserted. Optionally, such constructs encode a fusion KChAP which includes an N-terminal or C-terminal peptide or tag that simplifies purification of the expressed recombinant product. Representative examples of such tags include sequences which encode a series of histidine residues, the Herpes simplex glycoprotein D, or glutathione S-transferase.

Polynucleotides encoding KChAP are also useful for designing hybridization probes for isolating and identifying cDNA clones and genomic clones encoding KChAP, or for identifying cells and tissues containing KChAP transcripts. Such hybridization techniques are known to those of skill in the art. Sequence of polynucleotides that encode human or rat KChAP are also useful for designing primers for polymerase chain reaction, a technique useful for obtaining large quantities of cDNA molecules that encode KChAP. Preferably, the primers comprise 18-30 nucleotides, more preferably 19-25 nucleotides. Preferably, the primers have a G+C content of 40% or greater.

#### Cloning the C Terminal Region of a cDNA that Encodes Rat KChAP

A cDNA encoding the C terminal region of rat KChAP was isolated using the Yeast Two-Hybrid Library Screen and a rat brain cDNA library in the GAL4 activation domain vector, pGAD10, obtained from Clontech. In this procedure the entire coding sequence of Kv $\beta$ 1.2 (amino acids 1-408), which was used as bait for proteins that interact with Kv $\beta$ 1.2, was subcloned in frame into the GAL4 DNA binding domain vector, pGBT9 from Clontech after PCR-mediated addition of a 5' *EcoRI* site and a 3' *SalI* site. The yeast Y190 strain (with two reporter genes, *lacZ* and *HIS3*) was cotransformed simultaneously with Kv $\beta$ 1.2 pGBT9 and pGAD10 library DNA, and plated on synthetic medium lacking tryptophan (*trp*), leucine (*leu*), and histidine (*his*) plus 3-aminotriazole (25 mM) to prevent leaky transcription of the *HIS3* gene. After incubation for 8 days at 30°C, His colonies were screened for  $\beta$ -galactosidase activity by a

filter lift assay as outlined in Clontech protocols. Yeast DNA was isolated from colonies positive for both reporter genes using a phenol/glass-bead protocol (Clontech). Individual library plasmids were isolated after transformation of yeast DNA into chemically competent HB101 bacteria and growth on minimal medium lacking leucine. Individual pGAD10 recombinant plasmids were screened for interaction with Kv $\beta$ 1.2 by repeating the yeast two-hybrid assay in Y190 cells. One plasmid, designated herein as the "KChAP-Y plasmid", activated transcription of the reporter genes in cells co-transformed simultaneously with Kv $\beta$ 1.2 pGBT9, but did not activate transcription in control cells transformed with the KChAP-Y plasmid alone.

KChAP-Y plasmid cDNA was sequenced, and found to comprise a 1.78 kb insert encoding an open reading frame of 264 amino acids and 980 base pairs of 3' untranslated sequence including the poly (A<sup>+</sup>) tail. This insert or fragment is designated herein as "KChAP-Y".

#### Cloning of a Full-Length cDNA Encoding rat KChAP

A polynucleotide encoding KChAP-Y was used as a probe to obtain a full-length cDNA encoding KChAP. In this procedure, the <sup>32</sup>P labeled KChAP-Y insert was used to screen a rat brain cDNA library in  $\lambda$ gt10 from Clontech. One of the hybridizing clones contained an insert of 3.2 kb with a single open reading frame encoding a protein of 574 amino acids. The start methionine was identified as the first ATG downstream from three in frame stop codons.

The full length cDNA encoding KChAP, designated herein as the "KChAP gene" is shown schematically in Figure 1. The sequence of the KChAP gene is shown in Fig. 2 and set forth in SEQ ID NO: 1. Hydropathy analysis showed no putative membrane spanning regions in KChAP. The open reading frame of the KChAP gene predicts a protein of 574 amino acids and having the sequence shown in Fig. 2, SEQ ID NO: 2. The open reading frame of the KChAP gene is flanked by 219 base pairs of untranslated sequence on the 5' end, and 980 base pairs of 3' untranslated sequence. The 980 base pair sequence as indicated by the thin lines in Figure 1. The Kv $\alpha$ /Kv $\beta$  binding domain on the KChAP gene has been localized to the region which encodes amino acids W310 through L407. The Kv $\alpha$ /Kv $\beta$  binding domain has the amino acid sequence set forth in SEQ ID NO: 5. KChAP-Y extends from amino acid W310 of the full-length KChAP protein through the poly A tail at the 3' end.

### In Vitro Transcription and Translation of the KChAP Gene

Full-length KChAP cDNA was removed from pGBT9 with *EcoRI* and *Sall* and subcloned into a pCR3 vector which was modified to allow the cloning of *EcoRI/Sall* fragments in frame behind a *c-myc* tag. The KChAP fragment for subcloning was prepared by PCR to contain only a consensus Kozak sequence at the 5' end and a poly (A<sup>+</sup>) tail just past the stop codon at the 3' end to eliminate most of the 5' and 3' untranslated sequences in the expression construct. KChAP cRNA was prepared using the T7 mMESSAGE mMACHINE kit (Ambion) following linearization of the construct with *NotI*. cRNA for *c-myc*-KChAP was translated in vitro in a rabbit reticulocyte lysate to provide the fusion protein *c-myc*-KChAP.

### Cloning of a Full-Length cDNA Encoding Human KChAP

Reverse transcriptase and polymerase chain reaction (RT-PCR) techniques were used to clone a cDNA encoding human KChAP. The template was human brain poly A<sup>+</sup> RNA from Clontech. The reverse transcription reaction mixture was incubated for 1 hour at 42° C, and then heat inactivated.

PCR amplification was performed using the sense primer 5'ATGAAGATCAAAGAGCTTTACCGACG 3' , SEQ ID NO: 12 and the antisense primer 5'TCAGTCCAGGGAAATCATGACCG 3', SEQ ID NO: 13, which flank the start methionine and stop codon, respectively. The following reagent concentrations were used for amplification: 5% DMSO, 0.2 mM of each dNTP, 0.2 pmole/ µl of each oligomeric primer, Clontech's Advantage cDNA polymerase mix and buffer. The cycling protocol was as follows: one cycle at 94° C for 2 minutes; 35 cycles at 94° C for 15 seconds, 50° C for 15 seconds, and 72° C for 30 seconds; and one cycle at 72° C for 10 minutes.

The PCR product was gel-purified using Qiaquick Gel Extraction Kit from Qiagen, subcloned into PCRII from Invitrogen and sequenced using Sequenase from U.S. Biochemicals. The nucleotide sequence of the open reading frame, SEQ ID NO: 3, and the predicted amino acid sequence, SEQ ID NO: 4, encoded thereby are shown in Fig. 3.

### Increasing the Number of Kv2.1, 2.2, 1.3 and 4.3 Channels in Host Cells

#### A. Materials

Human Gu binding protein cDNA encoding the peptide spanning amino acids M49 to D645 was obtained by RT-PCR from human brain poly A<sup>+</sup> RNA and subcloned into pCRII (Invitrogen) for transcription. A Kozak sequence was included in the 5' oligo to facilitate translation in oocytes. cRNAs for Kv1 $\alpha$ -subunits were prepared as described in Majumder et al, (1995), FEBS Letts, 377, 383-389, and Wang et al. (1996), J. Bio. Chem., 271, 28311-28317. cRNA encoding Kv2.1 $\Delta$ N (in which the N-terminal 139 amino acids had been deleted) prepared was as described in VanDongen et al, (1990), Neuron 5, 433-443. HERG cDNA was obtained from Dr. M. Keating. Kv2.2 was obtained from Drs. S. Snyder and J. Trimmer. Rat Kv2.1 in pBluescript was linearized with *NotI* and cRNA was prepared with T7 polymerase. cRNA concentrations were estimated on denaturing agarose gels stained with ethidium bromide by comparison with RNA standards. cRNAs were mixed and injected into *Xenopus* oocytes as described in Majumder et al, (1995).

## B. Methods

*Xenopus* oocytes were co-injected with cRNA molecules encoding human KChAP protein or rat KChAP protein and with cRNA molecules encoding one of the following Kv $\alpha$  subunits: Kv1.2, Kv1.5, Kv3.1, Kv2.1, Kv2.2 and Kv4.3, or with cRNA molecules encoding Kir 2.2, HERG, and Kv2.1 $\Delta$ N. As a control, *Xenopus* oocytes were injected with cRNA molecules encoding the  $\alpha$ -subunits alone. For comparison *Xenopus* oocytes were co-injected with cRNA molecules encoding human Gu binding protein and the  $\alpha$ -subunits.

Whole oocyte currents were measured either two days after co- injection of *c-myc*-KChAP cRNA and cRNAs encoding Kv4.3, Kv1.2, Kv3.1, Kir 2.2, or aKv2.1 $\Delta$ N, or five days post-injection from oocytes injected with cRNAs encoding Kv2.1, Kv2.2, Kv1.5 or HERG plus *c-myc*-KChAP cRNA. Bath solution contained (in mmol/liter): 5 KOH, 100 NaOH, 0.5 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 100 MES, and 10 HEPES (pH 7.4). Solution containing 50 K<sup>+</sup> was prepared by replacing an equivalent concentration of Na<sup>+</sup>. Electrodes were filled with 3 M KCl and had a resistance of 0.3 - 0.6 M $\Omega$ . All recordings were made at room temperature. Linear leakage and capacity transient currents were subtracted (P/4 prepulse protocol) unless specified and data were low pass filtered at 1 kHz. pClamp software (Axon Instruments) was used for generation of the voltage-pulse protocols and data acquisition. Means  $\pm$  S.E.M. were calculated and were considered to be significantly different when P<0.05. Comparisons among multiple groups of

oocytes were performed by one-way ANOVA test and Student-Newman-Keuls post-hoc test (SKN test).

For Kv1.2, Kv1.5, Kv2.1, Kv2.1ΔN, Kv2.2 Kv3.1 and Kv4.3 channels holding potential was -80 mV. Peak (Kv4.3) or steady state (other channels) currents were measured at a test potential of +70 mV (5 or 50 K<sup>+</sup> in the bath) . Kir 2.2 and HERG currents were recorded with 50 K<sup>+</sup> in the bath at test potential to -100 mv with a pre-pulse to +20 mV.

The results indicated that co-injection of oocytes with cRNA encoding KChAP and encoding either Kv2.1  $\alpha$  subunit, or Kv2.2  $\alpha$  subunit, or Kv4.3  $\alpha$  subunit significantly increased the amplitude of Kv2.1, Kv2.2, and Kv4.3 currents as compared to oocytes injected with these respective  $\alpha$ -subunits alone. No change was observed in the currents of control oocytes or oocytes coinjected with cRNA encoding KChAP and cRNA encoding either Kv1.2  $\alpha$  subunit, or Kv1.5  $\alpha$  subunit, or Kv3.1 $\alpha$  subunit. The results also indicated that KChAP did not alter the kinetics or gating of Kv2.1, Kv2.2, or Kv4.3 channels.

Several hours after recording, the oocytes injected with cRNA molecules encoding KChAP and Kv2.2  $\alpha$  subunit were fixed in 4% paraformaldehyde. 50  $\mu$ m vibrotome sections were cut, and incubated for 2 hours in 1% BSA/PBS to block nonspecific binding sites. The sections were incubated at 4°C overnight in primary antibodies, that is an anti-Kv2.1  $\alpha$  subunit, rabbit polyclonal antibody, from Upstate Biotechnology, Inc.; and anti-c-myc, a mouse monoclonal antibody, from Boehringer Mannheim, Inc. The sections were washed, and incubated for 2 hours at room temperature in secondary antibodies, FITC-conjugated anti-rabbit for Kv2.1 and TRITC-conjugated anti-mouse for c-myc. Sections were examined with an Olympus BH-2 microscope for the appearance of fluorescence.

The FITC fluorescence, which indicates the amount of Kv2.1  $\alpha$  subunit, was much brighter at the oocyte surface in eggs expressing both Kv2.1 and KChAP as compared to eggs expressing the Kv2.1  $\alpha$  subunit alone. Thus, co-injection cRNA molecules encoding KChAP with cRNA encoding either Kv2.1, Kv2.2, or Kv4.3  $\alpha$  subunits increases the number of functional Kv2.1, Kv2.2 and Kv4.3 channels on the plasma membrane of cells as compared to cells injected with cRNA molecules encoding the Kv $\alpha$  subunits alone. By increasing the number of functional Kv2.1, Kv2.2 and Kv4.3 channels on the surface of a host cell, one can more

readily study the channels and more easily observe the effect of pharmaceutical agents on such channels.

#### Interaction of KChAP with Kv $\alpha$ and Kv $\beta$ Subunits.

The interaction of KChAP with particular Kv $\alpha$  and  $\beta$  subunits was examined using an indirect and direct procedure.

##### (a) Indirect Procedure for Monitoring Interaction of KChAP with Kv $\alpha$ and Kv $\beta$ subunits

In the indirect procedure, the yeast Matchmaker Two-Hybrid System Clontech and cDNA molecules encoding KChAP and KChAP-Y were used. The binding specificity between full length KChAP or KChAP-Y and the following Kv $\alpha$ , Kv $\beta$  and other K<sup>+</sup> channel subunit fragments were determined: Kv $\beta$ 1.2 (amino acids 1-408), Kv $\beta$ 1.2-N terminus (amino acids 1-79), Kv $\beta$ 1C (carboxyl terminal 329 amino acids of the Kv $\beta$ 1 subfamily), Kv $\beta$ 2 (amino acids 1-367); Kv1.1-N terminus (amino acids 1-168), Kv1.2 N-terminus (amino acids 1-124), Kv1.4 N-terminus (amino acids 1-305), Kv1.4 C-terminus (amino acids 562-654), Kv1.5 N-terminus (amino acids 1-248), Kv2.1 N-terminus (amino acids 1-168), Kv2.2 N-terminus (amino acids 1-185), Kv6.1 N-terminus (amino acids 1-209), Kir2.2 N-terminus (amino acids 1-86), and HERG N-terminus (amino acids 1-396). The ability of human Gu-binding protein (GBP) to bind to these fragments was also determined .

Protein-protein interactions were tested by co-transformation of the yeast host strain Y190 with a pGAD10 plasmid containing inserts encoding KChAP-Y, KChAP or GBP with a pGBT9 plasmid containing an insert encoding one of the K<sup>+</sup> channel protein fragments. pGBT9 is a GAL4 DNA binding domain [BD] vector. Co-transformants were plated on medium lacking *trp* and *leu* and grown for 2.5 days at 30°C. Yeast colonies were lifted to paper filters and assayed for  $\beta$ -galactosidase activity. Appearance of blue color within 8 hours was scored as a positive interaction between the proteins encoded by the two plasmids.

KChAP and KChAP-Y interacted with the fragments in a similar manner. KChAP interacted with the N-terminus of Kv $\alpha$ 1 subunits and the Kv $\alpha$ 2 subunits. KChAP also associated with the C terminus of Kv $\beta$ 1 and Kv $\beta$ 2 with no apparent interaction with the Kv $\beta$ 1.2 N- terminus. No binding was observed to the C-terminus of Kv1.4, nor to the N-termini of either HERG, Kir2.2, or Kv6.1. KChAP-Y interacted with the N-termini of Kv1.1, Kv1.2, Kv1.4,

and Kv1.5. No interaction was evident between the Kv1.4 C-terminus and KChAP-Y. KChAP-Y also interacted with the N-termini of Kv2.1 and Kv2.2, but not with the N-terminus of the electrically silent Kv2 partner, Kv6.1. Further specificity for a subset of Kv channels was apparent from the lack of interaction with the N-terminus of the inward rectifier K<sup>+</sup> channel, Kir2.2, and the N-terminus of the delayed rectifier K<sup>+</sup> channel, HERG. Thus, KChAP-Y both interact with the C-terminus of Kv $\beta$  subunits as well as the N-termini of Kv1 and Kv2  $\alpha$ -subunits.

The minimal KChAP sequence sufficient for Kv $\alpha$  and Kv $\beta$  binding was determined by expressing fragments of KChAP in yeast two-hybrid assays with the Kv fragments. The results indicated that the Kv $\alpha$  and Kv $\beta$  binding domain of KChAP is localized to a region between amino acids W310 and L407. Gu binding protein did not interact with any of the fragments. Thus, although KChAP shares 50% homology with GBP, interaction with Kv $\beta$  and Kv $\alpha$  subunits appears to be a unique feature of KChAP.

#### (b) Direct Procedure for Monitoring Interaction of KChAP and Kv $\alpha$ and Kv $\beta$ subunits

The direct procedure involved immunoprecipitation of protein complexes produced by *in vitro* translation of cRNA for *c-myc*-KChAP and cRNA for the  $\alpha$ -subunit Kv2.1. These cRNA molecules were translated *in vitro* either separately to produce individual proteins or together to produce complexes in rabbit reticulocyte lysates in the presence of <sup>35</sup>S-methionine using the Retic Lysate IVT kit (Ambion). For immunoprecipitation (IP), 10  $\mu$ l aliquots of each translation mixture were diluted into 1 ml IP buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA). To monitor the ability of the two proteins to associate after translation, 10  $\mu$ l aliquots of individual translates of Kv2.1 and *c-myc*-KChAP were mixed in 1 ml IP buffer prior to addition of antibody. IP was performed with two primary antibodies: anti-Kv2.1 polyclonal (1:100 dilution; Upstate Biotechnology, Inc.) or anti-*c-myc* monoclonal (1:400; Boehringer Mannheim). After addition of the primary antibody, the reactions were mixed gently overnight at 4°C. Immune complexes were collected on magnetic beads coupled to either anti-rabbit or anti-mouse secondary antibodies (Dyna, Inc.). After four washes in IP buffer, bound protein was eluted by boiling in SDS sample buffer, and analyzed on 10% polyacrylamide/SDS gels. The gel was fixed, soaked in Amplify (Amersham), and radiolabeled protein detected by fluorography.

The anti-Kv2.1 antibody immuno-precipitated complexes of Kv2.1 and *c-myc*-KChAP from translation reactions in which the two proteins were co-translated. The formation of a



complex between KChAP and Kv2.1 shows a direct interaction between the two proteins . No complexes of Kv2.1 and c-myc-KChAP were detected in samples in which the Kv2.1 and c-myc-KChAP were translated separately and mixed together before the addition of primary antibody. This result suggests that the association of KChAP with Kv2.1 occurs co-translationally since the mature proteins added after translation did not co-immunoprecipitate.

#### Altering the Effect of Kv $\beta$ 1-C on Kv1.5 Currents

*Xenopus* oocytes were co-injected with cRNA molecules encoding KChAP and cRNA molecules encoding Kv1.5  $\alpha$  subunit and Kv $\beta$ 1-C. Kv $\beta$ 1-C is known to interact with the Kv1.5  $\alpha$  subunit within the cell and to decrease Kv1.5 currents. As a control, *Xenopus* oocytes were injected with cRNA encoding the Kv1.5  $\alpha$  subunit alone or with cRNA molecules encoding Kv1.5  $\alpha$  subunit and Kv $\beta$ 1-C. Whole cell-currents were measured five days after injection. Holding potential was -80mV and pulses were from -70mV to +70mV in 10mV steps with 5mM K<sup>+</sup> in the bath solution.

Co-injection into cells of cRNA encoding KChAP and cRNA's encoding Kv1.5  $\alpha$  subunit and Kv $\beta$ 1-C blocked the effect of Kv $\beta$ 1-C on Kv1.5 currents.

#### Presence of KChAP in the Nucleus

Examination of the COS-7 cells and mouse L cells transfected with c-myc-KChAP cDNA and stained with FITC-labeled c-myc antibodies indicate that at least a portion of KChAP is located in the nucleus.

#### KChAP Expression in Rat Tissues

A <sup>32</sup>P-labeled riboprobe was prepared from a fragment of rat KChAP cDNA encoding the C-terminal 167 amino acids. This is the region of the KChAP protein which differs most from GBP. The riboprobe was used to probe A rat Multiple Tissue Northern blot (2 $\mu$ g poly A<sup>+</sup> RNA per lane) from Clontech. The hybridization was done overnight in NorthernMax hybridization buffer from Ambion at 68°C. The blot was washed with in 0.1 x SSC/0.1% SDS at 70°C. Autoradiography was performed for 5 hours at -70°C with Kodak Biomax MS film and intensifying screen. The results indicated that KChAP transcripts are most abundant in heart, brain, skeletal muscle, lung, spleen and kidney.

### Interaction of KChAP with the Tumor Suppressor Product p53

The interaction of KChAP with the tumor suppresser gene product p53 was examined using the yeast two-hybrid system. The results indicated that p53 binds to the Kv $\alpha$ /Kv $\beta$  binding domain of KChAP. Co-injection into *Xenopus* oocytes of cRNA molecules that encode p53 along with cRNA molecules that encode KChAP and the Kv2.1  $\alpha$  subunit suppressed the stimulatory effect of KChAP on formation of Kv2.1 channels.

### Testing the Effects of a Compound on Current Flow through Kv Channels

In order to test the stimulatory or inhibitory effect of a compound, particularly a pharmacological agent, on the flow of current through Kv channels, it is desirable to have a model system comprising a population of cells that have increased numbers of Kv channels on their cellular plasma membranes. Such model system is especially suitable for measuring small changes in current flow. Such model systems are prepared by co-injecting into host cells cRNA molecules encoding KChAP and cRNA molecules encoding a Kv $\alpha$  subunit. The encoding regions for KChAP and for the Kv $\alpha$  subunit may both be on a single cRNA molecule, or the encoding regions for KChAP and for the Kv $\alpha$  subunit may be on separate cRNA molecules. Preferably, the Kv $\alpha$  subunit is an exogenous Kv $\alpha$  subunit, i.e., the Kv $\alpha$  subunit is not normally expressed in the cell. Such model systems are especially useful for monitoring the effect of a compound on a particular Kv channel, i.e., the Kv channel formed by assembly of a plurality of the exogenous Kv $\alpha$  subunits. Thereafter, the cells are cultured for a time and under conditions which permit transformation of the host cells, i.e., expression of the co-injected cRNA molecules and assembly of Kv channels comprising the corresponding Kv $\alpha$  subunits.

The compound, which has been dissolved in a suitable carrier, is added to the culture medium of a test population of transformed host cells. Preferably, a plurality of concentrations of the compound are added to a corresponding plurality of test populations. The compound is also added to the culture medium of a control population of cells that have not been transformed, i.e., cRNA molecules encoding KChAP and the Kv $\alpha$  subunit are not injected into the cells. Thereafter, whole cell currents are measured using conventional techniques, such as for example, using a two microelectrode voltage-clamp technique and the gigaseal patch clamp technique. A difference between whole cell currents in the control population and the test populations is

indicative of a stimulatory or inhibitory effect of the compound on the Kv channels formed by the exogenous Kv $\alpha$  subunit. Such measurements are also used to determine the effective compound dosage.

- 5 While the invention has been described to some degree of particularity, various adaptations and modifications can be made without departing from the scope of the invention as defined in the appended claims.

2025074-021302